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Three T-Cell Epitopes Within the C-Terminal 265 Amino Acids of the Matrix Protein pp65 of Human Cytomegalovirus Recognized by Human Lymphocytes

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Although a T-cell response in human cytomegalovirus (HCMV)-immune individuals exists against the most abundantly expressed protein pp65 of the virus matrix, less is known about the determinants that evoke this response. The aim of the study was to identify regions within HCMV pp65 (ppUL83) that contain sequences for the cellular immune response by the use of three recombinant overlapping β-galactosidase pp65 fusion proteins (C74, C35, and C47), covering the C-terminal 265 amino acids of the entire pp65 sequence. Two T-cell epitope determinants were recognized by human lymphocytes of healthy, HCMV-seropositive, human leukocyte antigen (HLA)-typed individuals. One T-cell determinant (amino acids [aa] 303–388) was localized in the mid-region of the entire pp65 sequence and a second T-cell determinant (aa 477–561) within the C-terminal region. By fine mapping with synthetic hexadecamer peptides three T-cell epitopes were identified within these two regions: P10-I (aa 361–376) in the mid-region, P3-II (aa 485–499), and P6-II (aa 509–524) in the C-terminal region. Inhibition studies with monoclonal antibodies to HLA class I or class II revealed a class II restricted response to peptides P10-I or P6-II, respectively. P10-I responders shared the HLA-DR11 allele and P6-II responders the -DR3 allele. Therefore, these T-cell epitopes of HCMV pp65 might be presented in association with particular HLA class II alleles. ***J. Med. Virol. 52:68–76, 1997.***

KEY WORDS: human cytomegalovirus; T-cell response; matrix protein pp65; peptide antigens; HLA anchor motifs

INTRODUCTION

Infection with human cytomegalovirus (HCMV) is a frequent cause of serious illness for immunocompromised individuals such as bone marrow or solid organ recipients, HIV-infected patients or congenitally HCMV-infected children [Link et al., 1992, 1993; Gorensek et al., 1988; Jacobson and Mills, 1988; Gehrz, 1991].

In bone marrow transplantation (BMT) there is controversy currently concerning the effect of the humoral immunity against HCMV [Zaia, 1994]. However, the cellular defense mechanism against HCMV plays an important role. The generation of both HCMV-specific $CD8^+$ cytotoxic T lymphocytes (CTL) as well as $CD4^+$ T helper (T_H) cells is responsible for the recovery of BMT recipients from severe HCMV diseases [Reusser et al., 1991; Li et al., 1994; Walter et al., 1995]. In the murine model the murine cytomegalovirus (MCMV) immune control is principally governed by MCMV-specific $CD8^+$ CTL [Reddehase et al., 1987]. However, $CD4^+$ T-cells exert a compensatory function in situations where the $CD8^+$ CTL immune control is absent [Jonjic et al., 1990; Polic et al., 1995].

Several HCMV proteins have been identified as target antigens for human T-cell recognition during HCMV infection. These include the regulatory immediate early proteins IE1 (pUL123) [Alp et al., 1991] and IE2 (pUL122), the MHC class I homologue (gpUL18), the upper matrix protein pp71 (ppUL82) [He et al., 1995] and the glycoprotein gB (gpUL55) of the virus envelope [Liu et al., 1991] for lymphocyte proliferative or T_H cell responses. Fine mapping of T-cell epitopes has been done for IE1 and gB. Within IE1, six T_H cell

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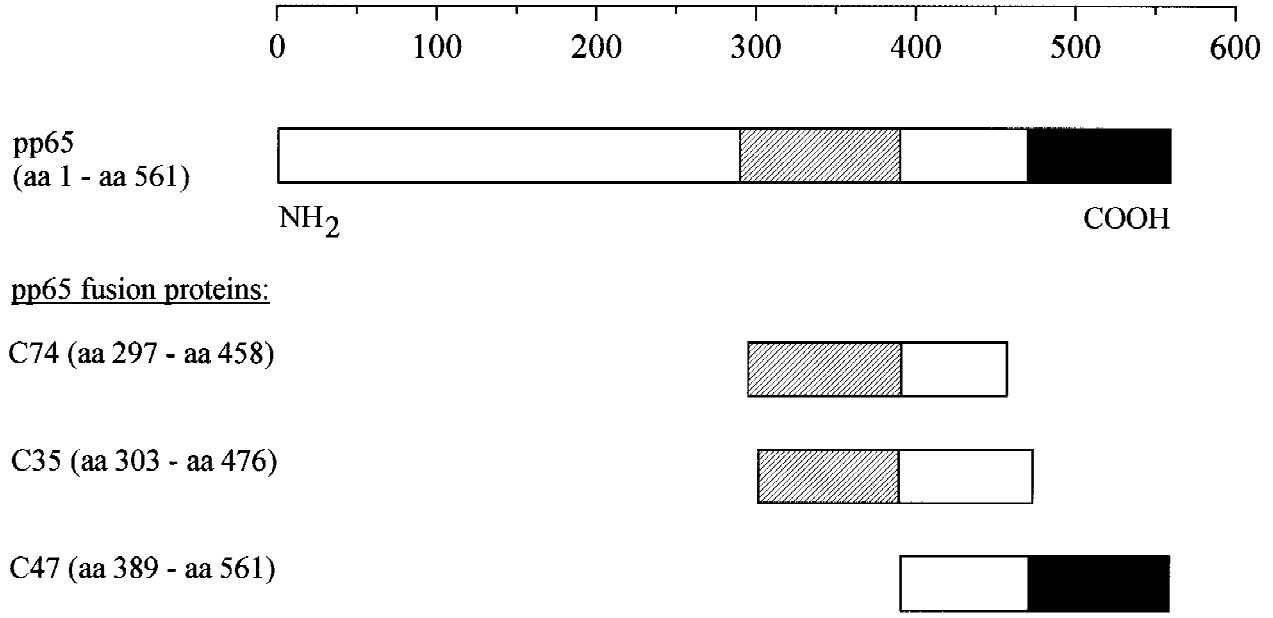

Fig. 1. Map of the primary structure of HCMV pp65 fusion proteins. The bars represent the entire pp65 sequence and the sequences of three pp65 subfragments contained in the pp65 fusion proteins C74, C35, and C47, respectively. The hatched areas represent the region characterized with synthetic peptides of series I. The filled bars represent the region characterized with peptides of series II.

and one CTL epitope are localized [Alp et al., 1991]. Foreign antigens are processed and presented to T-cells binding to human leukocyte antigen (HLA) molecules on the surface of cells, and allelic variation of HLA molecules influences the spectrum of antigens that individuals can respond to. Four discrete T_H cell epitopes are localized within gB, and the restricting HLA class II alleles for their presentation have been determined [Liu et al., 1993]. Cytotoxic T-cell responses have been described for IE1 [Rodgers et al., 1987], gpUL18 [Wronska et al., 1993], pp150 (ppUL32) [Riddell et al., 1993], and gB [Gilbert et al., 1993].

Likewise, the structural protein pp65 (ppUL83) of the virus matrix may be an important T-cell antigen. pp65 induces lymphocyte proliferative responses [Forman et al., 1985; Khattab et al., 1994]. In a comparative analysis of fourteen HCMV proteins it represented the dominant antigen recognized by T_H cells [Beninga et al., 1995]. For the cytotoxic T-cell response it has been shown that a pp65-specific $CD8^+$ CTL recognition of HCMV-infected cells occurs very early prior to de novo synthesis [Riddell et al., 1993; McLaughlin-Taylor et al., 1994], although this internal protein is abundantly expressed in the late phase of infection [Stinski, 1978]. The pp65-specific $CD8^+$ CTL precursor frequency dominates in comparison with the gB- and IE1-precursor frequency [Gilbert et al., 1993; Wills et al., 1995]. Hitherto, only one HLA-B35 restricted, cytotoxic T-cell epitope is reported for pp65 [Gavin et al., 1993].

The aim of this study was to identify regions within the matrix protein pp65 that contain T-cell epitopes. Using recombinant overlapping β-galactosidase pp65 fusion proteins and synthetic peptides three T-cell epitopes were identified. Furthermore, we tried to examine whether there is any association between particular HLA class II alleles and the recognition of these epitopes.

MATERIALS AND METHODS

HCMV Antigen Preparation

HCMV antigen was prepared from HCMV strain AD169-infected MRC-5 fibroblasts showing 90% cytopathic effect. Cells were freeze-thawed three times in phosphate-buffered saline (PBS) and centrifuged at 5,000*g* for 20 min at 4°C [Charpentier et al., 1986]. The supernatant was heat-inactivated at 60°C for 45 min [Alp et al., 1991] and a 1/20 dilution (referred to as HCMV antigen) was used for the stimulation of peripheral blood mononuclear cells (PBMC). Mock antigen was prepared from uninfected MRC-5 fibroblasts.

HCMV pp65 Fusion Proteins

To identify regions within HCMV pp65, relevant to the cellular immune response, we investigated the proliferative responses of PBMC to recombinant fusion proteins of pp65. Three overlapping β-galactosidase (β-gal) pp65 fusion proteins were chosen (Fig. 1), which are recognized by the humoral immune response [Landini et al., 1990; Lindenmaier et al., 1990]. The three pp65 fusion proteins (i) C74 (amino acids [aa] 297–458), (ii) C35 (aa 303–476), and (iii) C47 (aa 389–561) cover the C-terminal 265 amino acids of the pp65 sequence [Chee et al., 1990]. Cloning and preparation of these β-gal pp65 fusion proteins as well as preparation of β-gal lacking HCMV insert were previously described [Lindenmaier et al., 1990; Landini et al., 1990]. The fusion proteins were purified from inclusion bodies

by urea extraction and gel filtration chromatography on a sepharose 6B-CL column (Pharmacia, Freiburg, Germany).

Peptides

For fine mapping of T-cell epitopes two series of overlapping synthetic peptides were prepared (Fig. 1). Twelve 16-mer peptides of series I, each overlapping by eight amino acid residues, cover sequences common to the pp65 fusion proteins C74 and C35 (aa 289–392). Eleven 16-mer peptides of series II cover a selected part of the C47 sequence (aa 469–561). Peptide synthesis was done by standard Fmoc/tert-butyl chemistry with TBTU/NMM (O-benzo-triazolyl-N,N,N′,N′-tetramethyluronium terafluoro-borate/N-methylmorpholine) activation on Tentagel-SAC resin (Rapp Polymere, Tübingen, Germany) using a multiple synthesizer (Abimed Analysentechnik, Langenfeld, Germany), followed by deprotection and cleavage from the resin with trifluoroacetic acid containing 3% triisobutylsilane and 2% water. The crude peptides were purified by preparative high pressure liquid chromatography (HPLC). Peptide content in fractions was examined by analytical reversed phase HPLC and laser desorption mass spectrometry (MALDI-MS). Pure peptide fractions were concentrated, lyophilized and dissolved at 2 mg/ml in PBS.

Peripheral Blood Mononuclear Cells and Peptide-Induced T-Cell Lines

Peripheral blood mononuclear cells (PBMC) were obtained from 22 HCMV-seropositive (#1–#22) and 4 HCMV-seronegative (#23–#26), healthy blood donors by Ficoll-Isopaque (Biochrom, Berlin, Germany) gradient centrifugation. Cells were cultured in 96-well U-bottomed microtiter plates (Nunc, Roskilde, Denmark) in a total volume of 200 μl RPMI 1640 (Boehringer, Mannheim, Germany) per well supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% HCMV-seronegative, human AB serum (referred to as culture medium). Human leukocyte antigen (HLA) class I and II typing was performed by the blood bank of the Medical School Hannover using allotype-specific antisera.

Two peptide-induced, short-term T-cell lines were generated from PBMC of donor #2 and donor #11. For the generation of peptide-induced T-cell lines, 5×10^6 PBMC from donor #2 were initially stimulated with peptide 10 of series I and 5×10^6 PBMC from donor #11 with peptide 6 of series II, respectively. After 7 days 10 U/ml recombinant human interleukin-2 (Boehringer, Mannheim, Germany) was added for another 7 days.

Lymphocyte Proliferation Assay

1×10^5 PBMC/well were cultivated for 7 days in the presence of HCMV antigen, β-gal pp65 fusion proteins (5 μg/ml), or peptides (50 μg/ml). 1 μCi/well [^{3}H]thymidine (Amersham Buchler, Braunschweig, Germany) was added for the final 18 hr, followed by cell harvesting and liquid scintillation counting. Negative controls included: mock antigen (data not shown), β-gal, and medium alone.

The lymphocyte proliferative responses to the control antigen β-gal were usually comparable to cultures incubated with medium alone. Therefore, the stimulation indices (SI) were calculated from the mean counts per minute (cpm) of pentaplicate cultures with β-gal pp65 fusion proteins divided by the mean cpm with β-gal. Stimulation indices >3 were considered to be specific [Alp et al., 1991; Beninga et al., 1995]. Data of pentaplicate cultures of the stimulation assays with peptides are presented as the mean cpm ± standard error of mean (SEM).

For HLA-specific blocking of antigen presentation, monoclonal antibodies (mAb) to monomorphic determinants of HLA class I (W6/32; 165 mg/l) or DR (TAL.1B5; 53 mg/l) (Dako, Glostrup, Denmark) were dialyzed against PBS and added at 0.5 μg/ml to proliferation assays at the initiation of culture 30 min prior to adding peptides. Results were calculated as the percent inhibition of proliferation = {1 – [(cpm with peptide plus mAb) – (cpm with mAb)] divided by [(cpm with peptide) – (cpm with medium)]} × 100.

To determine the proliferative responses of peptide-induced T-cell lines, 2×10^4 T-cells were restimulated with peptides (50 μg/ml), β-gal pp65 fusion proteins (5 μg/ml), or HCMV as secondary antigens in the presence of 10^5 autologous, irradiated (3000 rad) PBMC for 3 days with [^{3}H]thymidine incorporation for the last 18 hr.

Cell Surface Phenotyping by Flow Cytometry

Peptide-induced cell lines were phenotyped for CD3, CD4, and CD8 expression by flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany) using 10 μl of either phycoerythrin or fluorescein isothiocyanate conjugated monoclonal antibodies (anti-CD3, anti-CD4, and anti-CD8; Becton Dickinson, Heidelberg, Germany).

RESULTS

Identification of T-Cell Responses to Subfragments of pp65

For an initial screening of potential T-cell epitope regions within HCMV pp65, the stimulatory capacity of the β-gal pp65 fusion proteins C74, C35, and C47 was investigated (Fig. 1). A summary of lymphocyte proliferative responses to the three fusion proteins obtained with the study group of 26 donors is shown in Table I: (i) 7/22 HCMV-seropositive individuals (#1–#7) were stimulated predominantly by C74 and C35 (S.I. range: 3.7–52.5). Donors #3 and #4 showed also a low response to C47. (ii) Further 7/22 HCMV-seropositive individuals (#8–#14) responded strongly to C47 (S.I. range: 3.3–16.8). Donor #14 was also slightly stimulated by C74.

TABLE I. Lymphocyte Proliferative Responses From HCMV-Seropositive and -Seronegative Donors to Fusion Proteins of the C-Terminal Part of pp65

Donors[a]	HLA haplotype[b]					Proliferative responses to pp65 fusion proteins[c]			HCMV[d]
						C74	C35	C47	
#1	A 1,11	B 51,8	DR 1,**11**(5)	DR 52	DQ 1,3	+++	+++	–	14.2
#2	A 2,33	B 51,44	DR **11**(5),8	DR 52	DQ 3,4	+++	+++	–	45.9
#3	A 3,32	B 13,61	DR 3,**11**(5)	DR 52	DQ 2,7(3)	+++	+++	+	282.4
#4	A 2	B 62,35	DR **11**(5)	DR 52	DQ 3	+++	+++	+	7.7
#5	A 1,74	B 51,13	DR **11**(5), 7	DR 52,53	DQ 2,7(3)	++	+	–	10.2
#6	A 2	B 51,7	DR 4,**11**(5)	DR 52,53	DQ 7(3)	+	+	–	14.6
#7	A 24,29	B 45,62	DR 4,**11**(5)	DR 52,53	DQ 7(3)	++	++	–	16.4
#8	A 1,2	B 7,44	DR 2,**3**	DR 51,52	DQ 1,2	–	–	++	4.7
#9	A 1,3	B 8,35	DR 1,**3**	DR 52	DQ 1,2	–	–	++	23.3
#10	A 1,3	B 8,49	DR **3,**4	DR 52,53	DQ 2,8(3)	–	–	+++	52.9
#11	A 1,26	B 8,27	DR 1,**3**	DR 52	DQ 1,2	–	–	+	13.2
#12	A 11,28	B 8,18	DR 2,**3**	DR 51,52	DQ 1,2	–	–	+	6.7
#13	A 1,3	B 8,35	DR 1,**3**	DR 52	DQ 1,2	–	–	+	26.1
#14	A 1,3	B 52,7	DR **17(3),**13(6)	DR 52	DQ 6(1),2	+	–	+	17.2
#15	A 2,28	B 14,62	DR 4	DR 53	DQ 8(3)	–	–	–	11.3
#16	n.d.	n.d.	DR 15(2),4	DR 51,53	DQ 6(1),7(3)	–	–	–	26.2
#17	A 3	B 35,47	DR 15(2)	DR 51	DQ 6(1)	–	–	–	32.7
#18	A 1,2	B 44,18	DR 4,7	DR 53	DQ 7(3)	–	–	–	8.1
#19	A 2,29	B 45,39	DR 15(2),4	DR 53	DQ 1,3	–	–	–	77.7
#20	n.d.	n.d.	DR 15(2),13(6)	DR 51,52	DQ 6(1)	–	–	–	12.4
#21	A 2	B 44,60	DR 2,4	DR 53	DQ 6(1),7(3)	–	–	–	4.4
#22	A 24,26	B 44,18	DR 4,12(5)	DR 52	DQ 7(3),8(3)	–	–	–	4.0
#23	A 32,28	B 44	DR 11(5)	DR 52	DQ 3	–	–	–	1.1
#24	A 2,26	B 13,37	DR 3,14(6)	DR 52	DQ 1,2	–	–	–	0.7
#25	A 1,3	B 7,60	DR 2,3	DR 51,52	DQ 1,2	–	–	–	1.0
#26	A 2,26	B 7,55	DR 2,14(6)	DR 52	DQ 6(1)	–	–	–	0.9

[a]Donors #1–#22 were classified as HCMV-seropositive and donors #23–#26 as HCMV-seronegative by the Virology Department of the Medical School Hannover.
[b]HLA class I and II haplotype analysis was performed using allotype-specific antisera. Allele super- or subtypes were determined for the A-, DR-, and DQ-locus; subtypes for the B-locus. Corresponding supertypes of subtypes from the DR- and DQ-locus are listed in parentheses. Shared alleles of pp65 fusion protein responders are indicated bold-faced. n.d. = Not done.
[c]Proliferation assays were set up incubating 10^5 PBMC from donors #1–#26 for 7 days in the presence of β-gal pp65 fusion proteins C74, C35, C47 (5 μg/ml), or HCMV antigen with [^{3}H]thymidine incorporation for the final 18 hr. Stimulation indices (SI) were calculated from the mean counts per minute (cpm) of β-gal pp65 fusion protein stimulated cultures/mean cpm of β-gal stimulated cultures; standard errors of means (SEM) were typically <10%. A SI >3 was considered to be specific. The positive response to recombinant, fusion proteins was graded from + to +++ according to the SI: +, SI 3–5; ++, SI 5–10; +++, SI > 10. No response: –, SI < 3.
[d]SI were calculated from the mean cpm of cultures stimulated with HCMV antigen/mean cpm of cultures with medium alone.

(iii) 8/22 HCMV-seropositive individuals (#15–#22) failed to recognize any of the three fusion proteins. Four HCMV-seronegative donors (#23–#26) responded neither to HCMV antigen nor to the fusion proteins.

Using these three overlapping pp65 fusion proteins, two T-cell reactive regions were identified (Fig. 1). The first region pp65-C74/C35 (aa 303–388) was recognized by individuals responding to C74 and C35, whereas the second region pp65-C47 (aa 477–561) was recognized by another cohort of donors responding nearly exclusively to C47.

Fine Mapping of T-Cell Epitopes With Peptides

Synthetic peptides representing sequences of the two regions identified were used to verify and characterize potential T-cell epitopes (Fig. 1). Results of lymphocyte proliferative responses from three individuals responding to three different peptides derived from the sequences of either region pp65-C74/C35 or region pp65-C47 are shown in Figure 2. The C74/C35 responder #3 recognized peptide P10-I (aa 361–376: PQYSEHPTFTSQYRIQ) derived from the pp65-C74/C35 region and to a lesser extent the adjacent peptide P11-I (aa 369–384: FTSQYRIQGKLEYRHT) (Fig. 2A). Two other C74/C35 responders (#1, #2) also showed higher proliferative responses to P10-I and significant but diminished responses to P11-I (data not shown). These results suggest that the epitope is located in the overlapping region of the two peptides (aa 369–376), but proliferative responses were rather contributed by adjacent amino acids in P10-I than by those in P11-I. The C47 responder #8 was stimulated by peptide P3-II (aa 485–499: PPWQAGILARNLVPMV) derived from the pp65-C47 region (Fig. 2B). A third epitope presented by peptide P6-II (aa 509–524: KYQEFFWDANDIYRIF) was recognized by the C47 responder #10 (Fig. 2C). Adjacent peptides to either P3-II or P6-II did not reveal proliferative responses.

The reactivity of pp65 fusion protein responders to these three peptides is shown in Table II. P10-I was recognized specifically by all seven C74/C35 responders (#1–#7). Of the C47 responders, individuals #9–#14 were stimulated by P6-II and two of them (#8, #14) by P3-II. Donor #14 was the only individual who responded to P6-II as well as to P3-II. P10-I was not able to induce a lymphocyte stimulation of C47 responders,

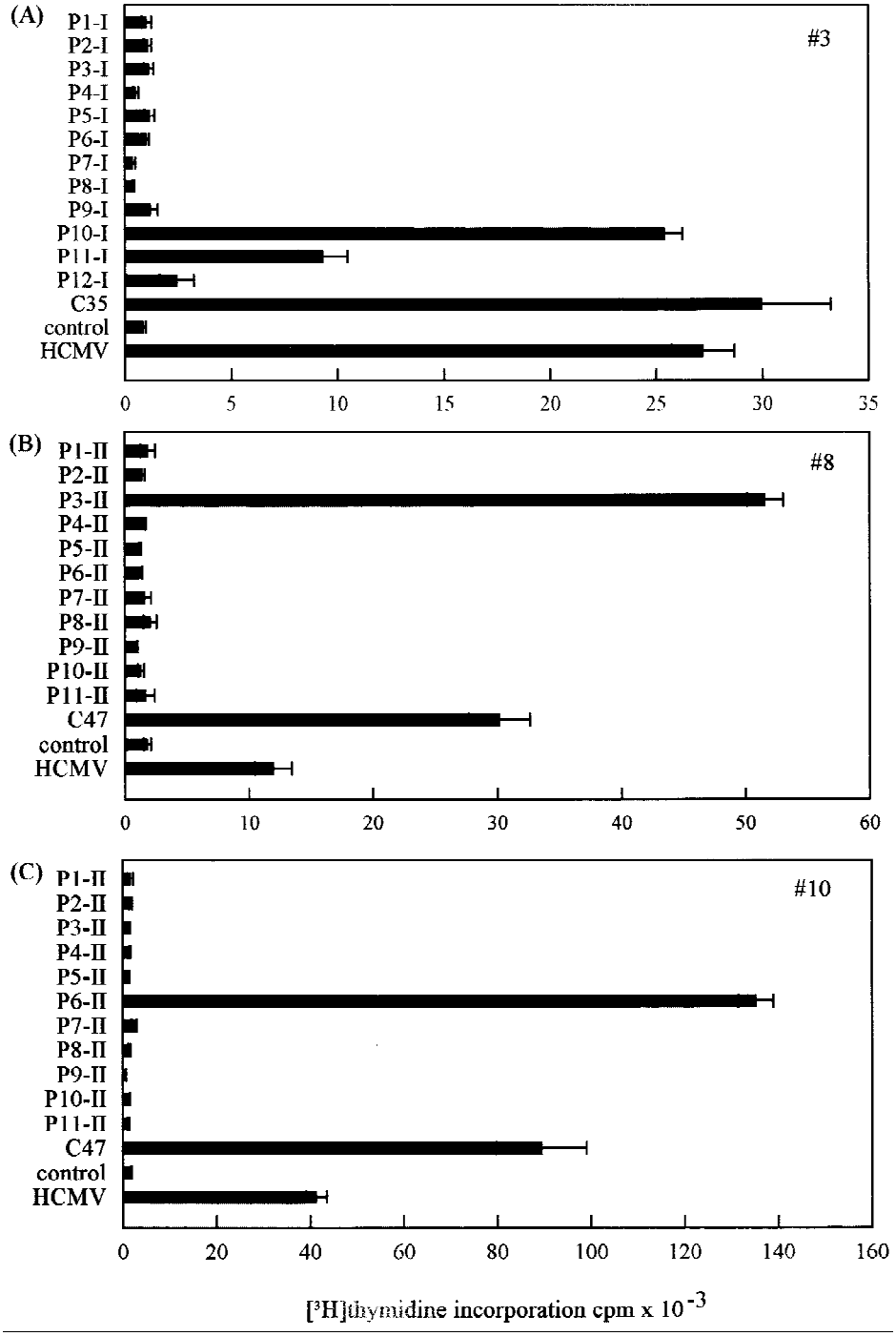

Fig. 2. Fine mapping of epitopes within the C-terminal 265 aa of pp65 by synthetic peptides. 10^5 PBMC of **(A)** donor #3, **(B)** donor #8, and **(C)** donor #10 were cultured for 7 days in the presence of antigens indicated with [^{3}H]thymidine incorporation for the last 18 hr. Peptides P1-I to P12-I represent 16-mers overlapping by 8 aa, which cover a C74/C35-specific region of pp65 (aa 289–392). Peptides P1-II to P11-II cover a C47-specific region (aa 469–561). Peptides were used at a concentration of 50 μg/ml, pp65 fusion proteins C35 and C47 at 5 μg/ml. Results are expressed as the mean cpm ± SEM of pentaplicate wells.

and P3-II or P6-II were not recognized by C74/C35 responders. Individuals who failed to respond to the pp65 fusion proteins also did not recognize any of the three peptides (representative data for subject #17 are listed).

The HLA-dependent recognition of peptides was studied using monoclonal antibodies reactive with HLA class I or DR. Lymphocyte proliferation of donor #1 induced by P10-I and of individual #11 by P6-II was significantly inhibited by a mAb to DR (87% and 75%, respectively), while the stimulation was only slightly reduced by a mAb to HLA class I (11% and 20%, re-

TABLE II. Lymphocyte Proliferation of pp65 Fusion Protein Responders to Peptides

Donors	HLA-DR[a]	Proliferative responses (cpm × 10^{-3} ± SEM)[b]				
		No antigen	HCMV	P10-I	P3-II	P6-II
#1	1,**11**	0.2 ± 0.0	53.7 ± 4.2	12.4 ± 0.6	n.d.	n.d.
#2	**11,**8	3.9 ± 0.7	39.6 ± 1.6	64.7 ± 3.1	5.1 ± 1.5	5.3 ± 1.6
#3	**3,11**	0.8 ± 0.1	27.1 ± 1.4	25.4 ± 0.4	1.1 ± 0.2	2.3 ± 0.4
#4	**11**	0.1 ± 0.0	9.2 ± 1.1	7.6 ± 0.8	0.8 ± 0.1	0.2 ± 0.0
#5	**11,**7	3.5 ± 1.9	16.8 ± 2.8	15.8 ± 1.9	4.3 ± 1.0	4.2 ± 0.7
#6	4,**11**	1.9 ± 0.1	28.8 ± 1.0	20.8 ± 2.9	n.d.	n.d.
#7	4,**11**	1.0 ± 0.0	62.4 ± 2.2	54.8 ± 1.3	2.4 ± 0.4	1.1 ± 0.2
#8	2,**3**	1.8 ± 0.3	48.1 ± 4.5	1.9 ± 0.1	44.7 ± 6.3	4.1 ± 0.6
#9	1,**3**	0.5 ± 0.0	47.9 ± 1.9	0.5 ± 0.0	1.5 ± 0.3	22.3 ± 5.2
#10	**3,**4	1.8 ± 0.0	41.3 ± 2.2	n.d.	1.3 ± 0.2	135.2 ± 3.6
#11	1,**3**	0.7 ± 0.1	41.7 ± 3.4	0.6 ± 0.0	2.0 ± 0.2	10.8 ± 0.9
#12	2,**3**	1.4 ± 0.1	21.5 ± 1.9	2.5 ± 0.1	2.4 ± 0.8	17.2 ± 1.5
#13	1,**3**	1.5 ± 0.3	10.1 ± 1.7	1.2 ± 0.2	2.1 ± 0.4	21.5 ± 2.3
#14	**17,**13	0.7 ± 0.2	14.2 ± 2.1	0.7 ± 0.0	16.4 ± 3.0	10.0 ± 2.8
#17	15	0.5 ± 0.1	16.6 ± 3.9	0.8 ± 0.2	0.8 ± 0.3	0.9 ± 0.2

[a]HLA haplotype analysis was performed as in Table I. Only the sub- or supertypes of the DR alleles are indicated. Shared alleles of responders are listed bold-faced.
[b]7-day lymphocyte proliferation assays were set up incubating 10^5 PBMC from donors #1–#22 in the presence of either HCMV antigen or peptides P10-I (PQYSEHPTFTSQYRIQ), P3-II (PPWQAGILARNLVPMV), and P6-II (KYQEFFWDANDIYRIF) at 50 μg/ml. Negative control: medium without HCMV antigen. [^{3}H]thymidine incorporation was included during the final 18-hr of culture time. Data are expressed as the mean cpm ± SEM of pentaplicate wells. Results were scored as significant when the mean cpm ± SEM in the presence of antigen was >three-fold the mean cpm ± SEM in the absence of antigen. Positive responses are underlined. n.d. = Not done.

spectively). These data indicated that the responding lymphocytes were MHC class II restricted, $CD4^+$ T-cells.

Recognition of Secondary Antigens by Peptide-Induced T-Cell Lines

Two short-term T-cell lines were generated after primary induction of PBMC from donor #2 with P10-I or from donor #11 with P6-II, respectively. Fluorescence flow cytometric analysis demonstrated predominantly a $CD3^+$ $CD4^+$ $CD8^-$ phenotype of these polyclonal T-cell lines (D2.P10-I: $CD3^+$ 93%, $CD4^+$ 80%, $CD8^+$ 8%; D11.P6-II: $CD3^+$ 61%, $CD4^+$ 50%, $CD8^+$ 9%). The P10-I induced T-cell line responded specifically to C35 and HCMV as secondary antigens (Table III). The P6-II induced T-cell line was restimulated by C47 and HCMV.

TABLE III. Proliferative Responses of Peptide-Induced T-Cells to Secondary Antigens

Cell line[a]	Primary antigens	Secondary antigens	Proliferative responses[b] (cpm × 10^{-3} ± SEM)
D2.P10-I	peptide P10-I	peptide P10-I	51.8 ± 1.1
		C35	22.7 ± 6.2
		HCMV	16.1 ± 2.5
		medium	1.0 ± 0.1
D11.P6-II	peptide P6-II	peptide P6-II	46.8 ± 3.3
		C47	35.4 ± 0.9
		HCMV	34.0 ± 1.1
		medium	2.3 ± 0.2

[a]The P10-I-induced T-cell line, D2.P10-I, and the P6-II-induced T-cell line, D11.P6, were generated by an initial 7 days stimulation of PBMC at 1 × 10^6/ml with peptides P10-I or P6-II (50 μg/ml), respectively, followed by an incubation with recombinant human interleukin-2 (10 U/ml) for further 7 days.
[b]2 × 10^4 T-cells plus 10^5 autologous, irradiated PBMC per well were restimulated for 3 days in the presence of peptides (50 μg/ml), pp65 fusion proteins (5 μg/ml), or HCMV as secondary antigens with [^{3}H]thymidine incorporation for the final 18 hr. Results are expressed as the mean cpm ± SEM of pentaplicate wells.

Association of Particular HLA-DR Alleles With T-Cell Proliferative Responses to Peptides

In an attempt to correlate the recognition of the three identified T-cell epitopes with the HLA-type of the donors, serological HLA class I and II typing was done for all individuals of the study group (Table I and II). The results indicated that particular DR alleles play a role in responsiveness to peptides. Donors #1–#7, recognizing the fusion proteins C74 and C35 as well as peptide P10-I, shared the DR11 allele. One individual (#4) was homozygous for DR11. Donors #9–#14, recognizing C47 as well as P6-II, shared the DR3 supertype allele. The DR3/DR11 expressing individual #3, a high C74/C35 responder and low C47 responder, was significantly stimulated by P10-I, barely by P6-II and not at all by P3-II. The DR3-expressing C47 responder #8 recognized only P3-II. Non-responders (donors #15–#22) to pp65 fusion proteins or peptides neither expressed DR11 nor DR3. These results suggest that the presentation of P10-I might be restricted by the DR11 allele, whereas the recognition of P6-II might occur in association with DR3.

DISCUSSION

After bone marrow transplantation the reconstitution of HCMV-specific cytotoxic T lymphocyte responses is the dominant defense mechanism in recovery from severe HCMV infections [Riddell et al., 1993; Walter et al., 1995]. The structural proteins pp65 and pp150 have been identified as the target antigens for $CD8^+$ CTL. But also the $CD4^+$ T helper cell response plays an important role, since a deficient $CD4^+$ T_H cell

TABLE IV. Putative HLA Class II Anchor Motifs Within Peptides P10-I and P6-II*

	Relative position									
	n	n + 1	n + 2	n + 3	n + 4	n + 5	n + 6	n + 7	n + 8	
DR11 motif 1[a]	**P**							**R,K**		
DR 11 motif 1 within P10-I (aa 361–376) PQYSEH	**P_{367}**	T	F	T	S	Q	Y	**R_{374}**	I	Q
DR11 motif 2[b]	**W,Y,F**			**M,L,V,I**		**R,K**				
DR 11 motif 2 within P10-I (aa 361–376) PQYSEHPT	**F_{369}**	T	S	Q	Y	**R_{374}**	I	Q		
DR11 motif 2 within P11-I (aa 369–384)	**F_{369}**	T	S	Q	Y	**R_{374}**	I	Q	G	KLEYRHT
DR3/DRw52 motif[c]	**F,I,L,V**			**D,N,Q,T**						
HLA-DR17 motif[d]	**L,I,F,M,V**			**D**		**K,R,E,Q,N**			**Y,L,F**	
DR17(3) motif within P6-II (aa 509–524) KYQE	**F_{513}**	F	W	**D_{516}**	A	**N_{518}**	D	I	**Y_{521}**	RIF

*Letters in bold indicate amino acids in single-letter code used as anchor residues.
[a]Based on Harris et al. [1993].
[b]Adapted from Hammer et al. [1993] and Hammer et al. [1994].
[c]Based on Loftus et al. [1995].
[d]Adapted from Malcherek et al. [1995] and Rammensee et al. [1995].

function influences the ability of patients to develop a HCMV-specific, endogenous CTL response [Reusser et al., 1991]. After adoptive immunotherapy HCMV-specific $CD8^+$ CTL responses decrease with time in patients in whom no development of endogenous, HCMV-specific $CD4^+$ T_H cell responses occurs [Walter et al., 1995]. In a comparative analysis of 14 individual HCMV proteins pp65 represented the dominant target antigen recognized by T_H cells of healthy, HCMV-seropositive individuals [Beninga et al., 1995]. It is therefore important to map epitope sequences, which elicit these T-cell responses.

Using three fusion proteins, which represent the C-terminal 265 aa of pp65, two discrete regions, containing potential T-cell epitopes, were identified. The first region pp65-C74/C35 (aa 303–388) was recognized by donors, responding to the pp65 fusion proteins C74 and C35 but not to C47. The second region pp65-C47 (aa 477–561) was recognized by another cohort of individuals, responding exclusively to C47. By fine mapping with synthetic peptides three discrete T-cell epitopes were identified within these two regions. The epitope P10-I (aa 361–376) is located in the mid-region of the entire pp65 sequence. Both, the epitopes P3-II (aa 485–499) and P6-II (aa 509–524) are located in the C-terminal region of the pp65 sequence. Peptide-induced T-cell lines recognized pp65 fusion proteins and HCMV as secondary antigens. Thus, these epitopes represent conserved structures of the pp65 protein.

So far, only one HLA-B35 restricted $CD8^+$ CTL epitope is localized in the N-terminal part of pp65 [Gavin et al., 1993]. McLaughlin-Taylor et al. [1994] have reported that target cells pulsed with cyanobromide (CNBr)-cleavage derived pp65 peptide fragments were recognized by HCMV-specific $CD8^+$ CTL. But in that study neither the exact localization and length of CNBr-pp65 fragments were specified, nor a different response pattern of the five investigated patients to particular CNBr-pp65 fragments was given.

Large exogenous proteins are processed generally by the endosomal pathway and presented by HLA class II molecules of antigen-presenting cells, which activate $CD4^+$ T-cells [Brodsky and Guagliardi, 1991]. Therefore, our strategy using exogenous recombinant β-gal pp65 fusion proteins would preferentially select T-cell epitopes recognized by HLA class II restricted $CD4^+$ T-cells. This was confirmed by the inhibition of lymphocyte proliferation in response to peptides P10-I or P6-II with a monoclonal antibody to HLA-DR, as well as by $CD3^+$ $CD4^+$ $CD8^-$ phenotyping of the two peptide-induced T-cell lines.

It was obvious that the T-cell epitopes identified were recognized in the context of different HLA alleles in a HLA-restricted manner. Peptide P10-I seems to be associated with the DR11 allele, since all seven P10-I responders shared this allele. Peptide P6-II may be associated with the DR3 allele, because six of eight DR3-expressing donors recognized this epitope. Investigating the T_H cell response to the entire pp65, Beninga et al. [1995] reported that three of their five responders expressed the DR11 subtype and another two either the DR3 supertype or its DR17 subtype. Possibly the epitopes identified could explain their results. However, in our study two DR3-expressing individuals were not stimulated by P6-II. One explanation for this failure could be that the PBMC cultures of these subjects contained very few precursor T-cells specific for P6-II, so that no significant responses to this peptide were observed. Analyzing the HLA restriction of T_H clones to gB peptides, Liu et al. [1993] noted that certain individuals expressing a restricting HLA allele exhibited no PBMC responses to the corresponding gB peptide. Second, in our HLA analysis the DR3 supertype was determined, which splits up into the subtypes DR17 and DR18. Such a subtype analysis was only done for individual #14, who expressed the DR17 subspecificity. Therefore, a further haplotype analysis of the two P6-II non-responders could demonstrate a possible expression of DR3 alleles (e.g., DR18), which might be not able to present peptide P6-II.

The sequences of the three identified peptides for proposed DR11, DR3, or DR17 anchor motifs, respec-

tively, were analyzed within a core region of nine amino acid residues (Table IV). Within peptide P10-I two possible DR11-binding motifs were found. Corresponding to the DR11-binding motif 1, suggested by Harris et al. [1993], $proline_{367}$ was N-terminally located in position n and $arginine_{374}$ at n + 7. In accordance with the DR11-binding motif 2 proposed by Hammer et al. [1993, 1994], $phenylalanine_{369}$ was located in position n and $arginine_{374}$ at n + 5. This second motif is common to peptides P10-I and P11-I and could explain the response of DR11 expressing donors to both peptides. For peptide P6-II, a possible DR17(DR3)-binding motif, suggested by Loftus et al. [1995], Malcherek et al. [1995], and Rammensee et al. [1995], was predicted with $phenylalanine_{513}$ in position n, $aspartate_{516}$ at n + 3, $asparagine_{518}$ at n + 5 and $tyrosine_{521}$ at n + 8. The location of the DR-binding motifs within P10-I and P6-II argues strongly for a presentation in association with the DR11 or DR3 allele, respectively. Within the sequence of peptide P3-II such binding motifs were not localized, and its restricting HLA allele(s) remain(s) to be elucidated.

Based on the evidence from this investigation and previous studies [Forman et al., 1985; Riddell et al., 1993; McLaughlin-Taylor et al., 1994], the pp65 peptide epitopes described might be helpful in the development of a HCMV subunit vaccine among other HCMV proteins such as gB [Gönczöl et al., 1990; Pass et al., 1995]. Alternatively, these peptides might be used as specific T-cell antigens for adoptive immunotherapy in BMT recipients to augment concurrently the HCMV-specific $CD8^+$ CTL response without requiring for an exposure to the infectious virus.

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